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Polymerase Chain Reaction for Detection of Invasive *Shigella flexneri* in Food

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The polymerase chain reaction (PCR) was used to amplify a 760-base-pair (bp) fragment with the 220-kbp invasive plasmids of enteroinvasive *Escherichia coli*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii*, and *Shigella sonnei* as templates. This PCR product was easily detected by agarose gel electrophoresis. A 210-bp *AccI-PstI* fragment lying within the amplified region was used as a probe in Southern hybridization blots and showed that the PCR-generated product was derived from the invasive plasmid. The application of PCR as a rapid method to detect enteroinvasive bacteria in foods was tested by inoculating lettuce with 10^4 *S. flexneri* cells per g in shigella broth base. Plasmid DNA was isolated from cultures of inoculated and uninoculated lettuce in broth after 0, 4, and 24 h of incubation. With the PCR, the 760-bp fragment was generated only from lettuce inoculated with *S. flexneri*, as shown by gel electrophoresis and confirmed both by Southern blotting and by nucleotide sequencing of the amplified region. Because the isolation of plasmid DNA, the performance of PCR, and gel electrophoresis all can be completed in 6 to 7 h, invasive enteric bacteria can be detected in less than 1 day.

Invasive *Shigella* species have been implicated in a number of recent outbreaks of food-borne disease (4), which are usually confirmed by clinical isolates before the contaminated food is found. In isolating and identifying these microorganisms from foods, several factors should be considered. Biochemical tests may not distinguish *Shigella* spp. from *Escherichia coli*, making it difficult to confirm the causative agent. Also, the number of shigellae present in the contaminated food may be low, affecting the ability of the shigellae to compete with the microflora of the food during selective enrichments.

Although colony hybridization (7) is useful for the detection of food-borne bacterial pathogens (9), concentrated homogenates must be plated on selective agar to allow target microorganisms to grow. Because enteroinvasive *E. coli* (EIEC) and *Shigella* spp. maintain some genetic information for virulence factors on a 220-kilobase-pair (kbp) plasmid (8, 23, 29), loss of the plasmid will result in noninvasive bacteria. Such strains would test negative in gene probe hybridization assays for plasmid-encoded virulence determinants. These difficulties (plasmid curing and low cell numbers) have led to a search for an alternative method to identify foods contaminated with invasive *Shigella* spp.

The polymerase chain reaction (PCR) (20, 21) provides a way of overcoming some of these difficulties. The selective and automated in vitro replication of a specified region of a known virulence gene can reveal the presence of a potentially pathogenic microorganism. This amplified DNA can be identified by agarose gel electrophoresis or by DNA dot blots with gene probes. In this study, we amplified a 760-bp region of a putative invasive gene in *Shigella* spp. and *E. coli* (K. A. Lampel, J. A. Jagow, and M. L. Troxell, in press) as a method for detecting enteroinvasive pathogens in foods. This method is sensitive, relatively rapid, and does not require selective enrichment, high cell numbers, or retention of plasmids during growth.

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MATERIALS AND METHODS

Strains. All enteroinvasive strains of *Shigella* spp. and *E. coli* were generously provided by T. L. Hale of the Walter Reed Army Institute of Research, Washington, D.C., and D. Yu, University of California at Los Angeles, Los Angeles. Strains used are listed in Table 1.

Primer sets for PCR. The 2.5-kbp *Hin*dIII fragment from EIEC (25) and the 2.5-kbp *Hin*dIII fragment from *S. flexneri* have been sequenced (Lampel et al., in press). The 2.5-kbp *Hin*dIII fragment from *E. coli* was subcloned into pUC19 and designated pIG500. The putative open reading frame predicted by the nucleotide sequence starts at base 108 and terminates at base 737. A set of primers was chosen to encompass most of this region. The primer KL1 (5′-TAATACTCCTGAACGGCG-3′, sense primer) is 18 bases in length and starts at base 149; KL8 (5′-TTAGGTGTCG GCTTTTCTG-3′, antisense primer) is 19 bases long and starts at base 897. The dissociation temperatures are 54°C for KL1 and 52°C for KL8 (26).

Primer synthesis and purification. Oligodeoxyribonucleotides were constructed by using a synthesizer (380B; Applied Biosystems, Foster City, Calif.). The instructions of the manufacturer were followed for synthesis, and completed oligomers were cleaved from the support by treatment with concentrated NH_4OH for 1.5 h. The reaction mixture was heated for 10 h at 55°C, and 1 ml of H_2O was then added to the trityl-DNA. Oligomers were purified by using an oligonucleotide purification cartridge (Applied Biosystems).

PCR conditions. Reactions were conducted by using a DNA amplification kit (GeneAmp; The Perkin-Elmer-Cetus, Corp., Norwalk, Conn.) as recommended by the manufacturer, with the following exceptions. All reactions were carried out by using 30 cycles, each consisting of 1 min at 94°C, 1.5 to 2.0 min at 55 to 65°C, and 1.5 to 2.0 min at 72°C. For primers KL1 and KL8, 10 pmol of DNA per reaction and 1.0 mM magnesium were used. The amount of template DNA added was usually between 1 and 5 ng of total DNA (3 to 15 pg of the target region when purified plasmid DNA was used).

TABLE 1. *E. coli* and *Shigella* strains used to test specificity of PCR

Strain[a]	Relevant genotype[b]	Reference or source
E. coli	*vir*$^+$, 0123	8
S. flexneri 270	*vir*$^+$, M90T	23
S. flexneri 354	*vir* mutant, EDL 945	D. Yu
S. sonnei	*vir*$^+$	22
S. dysenteriae	*vir*$^+$	6
S. boydii	*vir*$^+$	WRAIR[c]

[a] *S. flexneri* 354 was provided by David Yu. All other strains were provided by T. L. Hale.
[b] Strains were tested for invasiveness (*vir*) by the method of Sereny (24).
[c] WRAIR, Walter Reed Army Institute of Research strain collection.

Plasmid isolation. Total plasmid DNA was isolated from 1 ml of overnight cultures of enteroinvasive enteric bacteria grown in Luria-Bertani broth at 37°C or from 1 ml of the sample food (lettuce) by a modified alkaline denaturation protocol (1). DNAs were stored in distilled H_2O at 4°C until needed.

Materials. Restriction endonucleases, T4 kinase, and a random primer kit were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and used according to the instructions of the manufacturer. *Taq* DNA polymerase was obtained from Perkin-Elmer-Cetus. A Sequenase version 2.0 kit was purchased from U.S. Biochemicals (Cleveland, Ohio). Radioisotopes were obtained from ICN Radiochemicals (Irvine, Calif.).

Electrophoresis. Gels were composed of 1% agarose (Bethesda Research Laboratories) in 1× TBE buffer (14) and 100 ng of ethidium bromide per ml (Sigma Chemical Co., St. Louis, Mo.). A 20-μl sample of each mixture was loaded, and the gels were run for 1 h at 150 V. Total plasmid DNA was separated by electrophoresis under the same conditions but for 3 h.

Lettuce seeding. Lettuce (25 g) was aseptically added to a 500-ml Erlenmeyer flask containing 225 ml of Shigella broth (15) without novobiocin. An overnight *S. flexneri* 270 culture was diluted and added to the broth to yield a final concentration of approximately 10^4 cells per ml and incubated at 37°C with shaking (75 rpm). A similar sample with 25 g of lettuce was incubated as described above but was not inoculated with *S. flexneri*. At 0, 4, and 24 h, 10 ml of broth was removed, diluted, and plated onto MacConkey and Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates and incubated at 37°C overnight. Colonies from each plate were blotted on no. 541 filter papers (Whatman, Inc., Clifton, N.J.) (13) and prepared for colony hybridizations by denaturing the DNA in 2 ml of 0.5 N NaOH for 7 min and then washing the filters twice for 2 min each with 2 ml of 1.0 M Tris hydrochloride (pH 7.4), and once for 2 min with 2 ml of 1.0 M Tris hydrochloride (pH 7.4)–1.5 M NaCl. The filters were allowed to air dry.

At each time point, 1.0 ml from each broth culture was removed and centrifuged at 6,000 × *g* for 30 s. Total plasmid DNA was isolated by using an alkaline denaturation protocol (1).

DNA labeling. Oligonucleotides were end labeled by the procedure of Maniatis et al. (14).

Colony hybridizations. Labeled probe KL1 was hybridized against DNAs from bacterial colonies fixed to Whatman no. 541 filter paper. Hybridizations were conducted as described by Hill et al. (12) but with no preincubation of filters. After hybridization overnight (20 to 24 h), filters were washed twice in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 54°C, dried, and exposed to X-ray film at −70°C.

Southern blots. DNAs were transferred from agarose gels to nitrocellulose supports by using alkaline denaturation and capillary action (5). Hybridization was carried out as described by Hill and Payne (11). After overnight incubation with about 10^6 cpm of ^{32}P-labeled probe (KL1 or 210-bp *Acc*I-*Pst*I fragment) at 37°C in 50% formamide and 6× SSC, the nitrocellulose sheets were washed for 5 min at room temperature in 2× SSC–0.1% sodium dodecyl sulfate. Next, the sheets were washed twice for 20 min each time in 0.1× SSC, 0.1% sodium dodecyl sulfate at 54°C, and dried; X-ray film was exposed at −70°C for 24 h.

RESULTS

PCR-generated fragments from enteroinvasive enteric bacteria. Figure 1 shows a partial restriction map of the 2.5-kbp *Hin*dIII fragment from the 220-kbp invasive plasmid of EIEC and *S. flexneri*. The location of the PCR oligodeoxyribonucleotide primers and probes and the region amplified are indicated. Plasmids isolated from EIEC and *Shigella* spp. were subjected to PCR, and the products were analyzed by agarose gel electrophoresis (Fig. 2A). DNAs transferred to nitrocellulose filter paper were hybridized against the 210-bp *Acc*I-*Pst*I probe (Fig. 2B) isolated from pIG500. Those strains that carry the invasive plasmid generated the expected 760-bp fragment hybridized to the internal probe. The avirulent strain, *S. flexneri* 354, does not carry the template plasmid DNA for PCR and therefore did not produce the 760-bp fragment. The 210-bp probe hybridized to the 0.5-kbp *Pst*I-*Hin*dIII fragment of pIG500 (Fig. 2B).

Lettuce seeded with *S. flexneri*. Two flasks of Shigella broth (225 ml) with 25 g of lettuce, one inoculated with 10^4 *S. flexneri* cells per g and the other uninoculated, were shaken at 37°C for 24 h. Samples (10 ml) were removed at 0, 4, and 24 h, diluted, and plated onto MacConkey or Trypticase soy agar plates. Using probe KL1, colony hybridizations were carried out to determine the presence of any enteroinvasive bacteria in the lettuce. The number of bacteria counted and the hybridization results are shown in Table 2. The *Shigella* probe did not hybridize with DNA isolated from the uninoculated lettuce broth at any time.

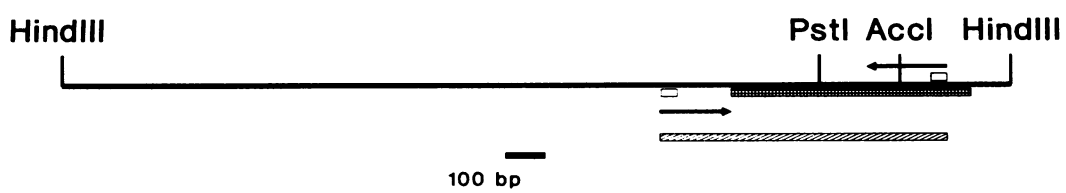

FIG. 1. Partial restriction enzyme map of 2.5-kbp *Hin*dIII fragment. Location of oligodeoxyribonucleotide primers (▭), direction of extension by *Taq* DNA polymerase (arrows), open reading frame (▩), and the 760-bp amplified DNA by PCR (▨) are shown. Restriction sites *Pst*I and *Acc*I are within the amplified region.

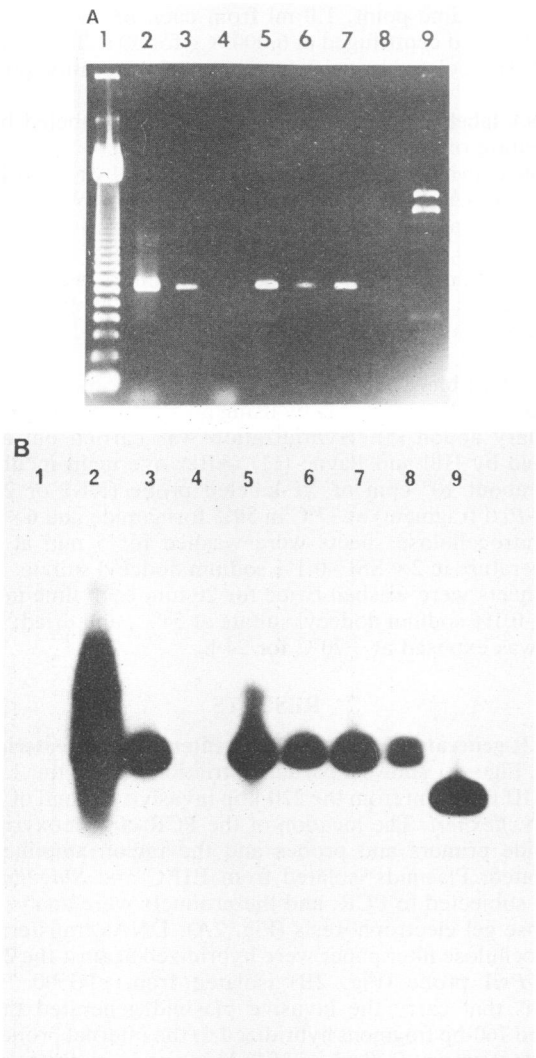

FIG. 2. Testing PCR for specificity. (A) Gel electrophoresis pattern of PCR products from enteroinvasive bacteria. PCR used the invasive plasmid as template from cesium chloride gradient-purified plasmid from *S. flexneri* 270 (lane 2), *S. flexneri* 270 (lane 3), *S. flexneri* 354 (lane 4), *S. dysenteriae* (lane 5), *S. sonnei* (lane 6), *S. boydii* (lane 7), and EIEC (lane 8). Lane 1 is 123-bp ladder standard; lane 9 is pIG500 digested with *Pst*I and *Hin*dIII. (B) Hybridization of Southern blot from gel in panel A using a 210-bp *Acc*I-*Pst*I probe.

At 0, 4, and 24 h, samples (1.0 ml) were removed from both flasks. Total plasmid DNAs were isolated and subjected to PCR. The amplified products were initially examined by gel electrophoresis to determine if the 760-bp segment was generated (Fig. 3A). To confirm further that the fragment of this size was actually DNA derived from the 2.5-kb *Hin*dIII region of the invasive plasmid, these DNAs were hybridized against the 210-bp *Acc*I-*Pst*I fragment that is within the expected amplified product. The DNA generated from the lettuce inoculated with *S. flexneri* was 760 bp long and did hybridize to the 210-bp *Acc*I-*Pst*I probe (Fig. 3B). To obtain additional evidence, the PCR-generated fragment was subjected to digestion with *Pst*I. The *Pst*I site lies near the middle of the generated PCR product (Fig. 1). Upon digestion with *Pst*I, the DNA was cut into two fragments of the expected sizes, 360 and 400 bp, as seen by gel electrophoresis through a 1% agarose gel (data not shown). The 760-bp DNA fragment was isolated from the 4-h culture and purified by electroelution; the nucleotide sequence was determined with KL1 as primer. A partial reading confirmed that the generated sequence matched the template DNA of the large invasive plasmid.

TABLE 2. Bacterial counts of uninoculated lettuce and lettuce seeded with *S. flexneri*

Time (h)	Lettuce inoculation[a]	Plate count/ml on:		No. of plasmid-positive *Shigella* spp. (CFU/ml)[b] on:	
		MacConkey agar	TSA[c]	MacConkey agar	TSA
0	−	1.8×10^4	5.7×10^4	0	0
4	−	1.0×10^5	1.3×10^5	0	0
4	+	1.1×10^7	1.1×10^7	9.8×10^6	9.8×10^6
24	−	1.2×10^9	1.2×10^9	0	0
24	+	2.1×10^9	2.8×10^9	1.3×10^9	1.4×10^9

[a] +, Inoculated; −, uninoculated.
[b] Determined by colony hybridization with ^{32}P-labeled oligodeoxyribonucleotide probe specific for the invasive plasmid.
[c] TSA, Trypticase soy agar.

DISCUSSION

Although outbreaks of disease caused by food-borne *Shigella* spp. are common, this pathogen is not easily isolated from foods. Frequently, the causative agent is found first in clinical specimens before an outbreak is recognized. To determine what food is implicated as the vehicle in an outbreak of shigellosis, methods for isolating *Shigella* spp. from foods need to be improved. Ideally, a protocol that identifies solely invasive *Shigella* spp. is preferred. Because the infective dose (10^1 to 10^4 cells) is relatively low (2), isolation methods must be sensitive enough to detect small

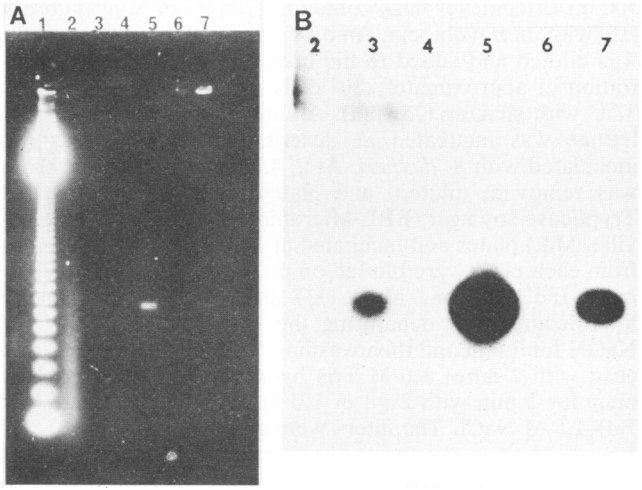

FIG. 3. Testing of lettuce for virulent *S. flexneri* by using PCR. (A) Gel electrophoresis of 760-bp PCR products derived from lettuce seeded with *S. flexneri*. Plasmid DNA extractions were performed on samples from unseeded lettuce at 0 h (lane 2), 4 h (lane 4), and 24 h (lane 6) and from lettuce seeded with *S. flexneri* at 0 h (lane 3), 4 h (lane 5), and at 24 h (lane 7). Lane 1 is 123-bp ladder standard. (B) Southern blot of PCR products from gel in panel A to 210-bp *Acc*I-*Pst*I probe. Lanes 2 through 7 are hybridizations of total plasmid DNAs from unseeded (lanes 2, 4, and 6) and *S. flexneri*-seeded (lanes 3, 5, and 7) lettuce at 0, 4, and 24 h, respectively.

numbers in foods. For these methods, cells are usually grown in broth for up to 24 h, and shigellae may therefore be overgrown by the indigenous microflora.

DNA probes have been targeted against the large invasive plasmid of enteroinvasive bacteria (25, 28, 30; J. A. Jagow and K. A. Lampel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, P14, p. 321); however, the plasmid is known to be unstable even after long storage in culture collections (3), and plasmids can be easily lost after overnight culture under nonselective or competitive conditions. To detect a particular gene by colony hybridization, 10^5 to 10^6 copies of the target sequence are needed. PCR can compensate for the low number of cells and plasmid instability by increasing the amount of target, even from a small number of templates.

PCR technology has been used to detect another foodborne pathogen, *E. coli*. Regions of the heat-labile enterotoxin gene have been amplified by PCR (18) to detect enterotoxigenic strains of *E. coli* from clinical specimens (17).

Many enrichment protocols fail to detect strains of virulent bacteria present in foods at low levels. Such strains, especially those pathogenic for humans, do not compete effectively in physiologically demanding enrichments and can be overgrown by nonpathogenic members of the same genus or species (10, 16). Strains may lose plasmids that harbor virulence genes, and the result is the recovery of nonpathogenic strains that do not hybridize to plasmid-associated virulence gene probes.

The PCR provides a mechanism to circumvent these difficulties by permitting the specific enzymatic replication of target genes at rates higher than that of biological amplification. Because cell growth and replication are not prerequisites for this method, the detection and identification of injured cells should pose no additional difficulties.

Theoretically, PCR can detect a single copy of target DNA so that the sensitivity of this method is limited by the recovery of the target sequences (e.g., plasmid) and the efficiency of the reaction. A limiting factor in detecting one *Shigella* organism would be isolating a template DNA from that one bacterium from the food. With appropriate treatments and a combination of centrifugation and filtration, a suitable DNA may be prepared for use as a PCR template from 10 g or more of food.

The most serious potential disadvantage of PCR-based assays is that secondary priming sites may yield a montage of amplified sequences or a fragment equal in size to the expected PCR product. Different temperatures are required for denaturing target strands, annealing primers, and optimizing *Taq* replication efficiency. Therefore, it is not possible to carefully control hybridization stringency during the entire course of the reaction. Because some low-stringency primer annealing and subsequent extension may occur during the initial temperature increase to reach the 94°C nuclease inactivation period, reaction tubes should be kept on ice until the heating block has reached this temperature. Other amplification products may result if the Mg^{2+} concentration is not optimized (19) or if the annealing temperature is too low. In some cases, the annealing temperature can be increased to the dissocation temperature of the primers.

Although injured cells in the food may not be a problem with the PCR, dead cells can yield positive results if the segment of DNA including the primer sites is intact. Therefore, pasteurized products may consistently yield positive tests by PCR, but viable organisms may not be recovered. The presence of dead cells is useful information regarding the quality of foods, but such products do not constitute a health hazard. The effect of false-positive results may be reduced by requiring a brief growth step (3 to 5 cell doublings) followed by a dilution before PCR is conducted. DNA that could not be biologically duplicated would be diluted out. This method would be useful, however, only if the level of dead cells is relatively constant.

After a recent shigellosis outbreak caused by contaminated lettuce, Davis et al. (4) inoculated shredded lettuce with *S. sonnei* (1.4×10^3 cells per g). After 12 h of incubation at 22°C, 1.8×10^6 cells per g were obtained, indicating that *Shigella* spp. can grow on lettuce with a doubling time of less than 2 h. They also showed that *Shigella* spp. can survive refrigeration for at least 1 week. Therefore, PCR should be quite efficient in increasing the number of target sites for DNA probes if the shigellae maintain the large invasive plasmid. In situations in which the shigellae do not survive in foods for any appreciable length of time, PCR can amplify the target region even when cells cannot be resuscitated. J. L. Smith and B. J. Dell (Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, P21, p. 322) and Tollison and Johnson (27) studied the recovery of heat-injured shigellae on various selective media containing Desoxycholate (BBL) and bile salts and reported that these injured cells may not always be cultivated.

Two other parameters need to be explored. In lettuce seeded with shigellae, the lowest number of bacteria that can be used to successfully amplify the 760-bp region must be determined. Another limiting factor is the isolation of plasmids from a low number of seeded bacteria.

In lettuce seeded with 10^4 invasive *S. flexneri* cells per g, a positive result was obtained immediately after inoculation and a stronger reaction product was obtained after 4 h of incubation. This assay, performed by using PCR followed by gel electrophoresis, required less than 1 day and involved no radioactivity.

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